Equine Veterinary Journal

Equine Veterinary Journal ISSN 0425-1644
DOI: 10.1111/evj.12308

Grayson-Jockey Club Research Foundation: Review Article

Stallion fertility: A focus on the spermatozoon

D. D. VARNER*, Z. GIBB† and R. J. AITKEN†

Department of Large Animal Clinical Sciences, College of Veterinary Medicine and Biomedical Sciences, Texas A&M University, College Station, USA
†School of Environmental and Life Sciences, Discipline of Biological Sciences, University of Newcastle, Callaghan, New South Wales, Australia.

*Correspondence email: dvarner@cvm.tamu.edu; Received: 21.01.14; Accepted: 03.06.14

Summary

Stallion fertility is a vast subject, with a wide array of permutations that can impact reproductive performance in either positive or negative ways. This review is intended to address a mere segment of the male fertility issue, but the very essence of the male contribution to fertilisation, that of the spermatozoon. Spermatozoal ultrastructure and form-to-function are detailed and spermatozoal metabolism is discussed, with specific reference to distinctive characteristics of stallion spermatozoa. Lastly, methods for assessment of spermatozoal function are considered, with emphasis on spermatozoal motility, the acrosome reaction and spermatozoon–oocyte interactions. Closing comments address the need for development and standardisation of molecular-based assays for use with spermatozoa of stallions whose subfertility cannot be explained with conventional tests.

Keywords: horse; stallion; sperm; spermatozoa; function; evaluation

Introduction

Unlike males of other agricultural species, stallions become sires based primarily on pedigree, performance record and conformation, with little consideration of reproductive soundness. The horse industry abounds with sires whose fertility is less than that considered standard for other agricultural animals. Added to this quandary is the circumstance of increased breeding duty, especially in the Thoroughbred industry, where popular stallions may breed over 4 times as many mares during a conventional 5-month breeding season than was customary only a couple decades ago. Furthermore, numerous stallions are nowadays subjected to dual-hemisphere breeding schedules whereby they are exposed to extraordinary travel stresses and increased breeding demands.

With this introduction, the intent of this article is to acquaint the reader with the current knowledge of fertility and spermatozoal function in stallions, as well as areas that warrant intensified research. Most research addressing spermatozoa does not use stallions as a model. As an example, a query for keywords in PubMed, reveals logged entries for equine spermatozoa are only 3% of that logged for human spermatozoa and only 10% of that logged for mouse spermatozoa. As such, we will draw from information garnered from other species in many instances, appreciating that direct extrapolation of these findings to stallion spermatozoa may be precarious.

Fertility of stallions can be negatively impacted by a wide and diverse assortment of conditions such as behavioural disorders, disturbances of mating ability, ill health or reproductive problems unrelated to spermatozoal function. Given the breadth of maladies that can impact fertility, our focus will be strictly on some features of spermatozoal structure and function as they relate to fertility.

Spermatozoal structure

A spermatozoon has but one role to fill, that of fertilisation of an oocyte – and billions of spermatozoa are generally present in an ejaculate to fulfil that mission. Why are such a large number of spermatozoa required to complete this task? The answer stems partially from the fact that fertilisation *in vivo* is an inherently inefficient process. Only 0.0007% of spermatozoa deposited into the uterus of the mare gain access into the oviductal luminae where fertilisation occurs [1]. Spermatozoa would appear to be relatively simple cells, being composed of a head and an attached flagellum and stripped of most organelles and cytoplasm during their formation in the testis and maturation in the epididymis. This 'simple' design can be misleading, as a spermatozoon must be extremely sophisticated and adaptable to achieve the task of fertilisation, requiring a series of highly coordinated cellular-level and molecular-level functions to be successful in its mission.

The spermatozoon is classically divided anatomically into a head and a flagellum (or tail). The head contains the *nucleus*, an overlying *acrosome* and a reduced complement of cytosolic elements. The head can be subdivided into an acrosomal region, equatorial segment, post acrosomal region and *posterior ring*, which demarcates the junction between the head and flagellum. The posterior ring is the site of plasma membrane anchoring to the *nuclear envelope*, and is thought to produce a tight seal that separates cytosolic components of the head and flagellum. The flagellum can be subdivided into a *connecting piece*, *middle piece* (or *midpiece*), *principal piece* and *end piece* (Fig 1). These various parts of the spermatozoon are surrounded by a common plasma membrane; however, the composition of the plasma membrane can be subdivided into regional domains that impact its multiple functions, such as: sperm–oviductal adhesion; penetration of the cumulus–oophorus matrix; sperm–zona adhesion; the acrosome reaction; acquisition of activated motility and hyperactivated motility; and sperm–oocyte adhesion and fusion.

The equine spermatozoon has similar general structures to that described for the bull, ram, boar, ram, dog and man. The average length of an equine spermatozoon is 61–86 μm [2–4]. The size of an equine spermatozoon, relative to body size, is remarkably smaller than that of some other species, such as the mouse, rat, hamster and honey possum, where spermatozoal lengths are 123, 190, 189 and 356 μm, respectively. The average length of a human spermatozoon is 57 μm [2].

The equine spermatozoon head is defined as spatulate-shaped, in contrast to the falciform-shaped spermatozoal heads characteristic of

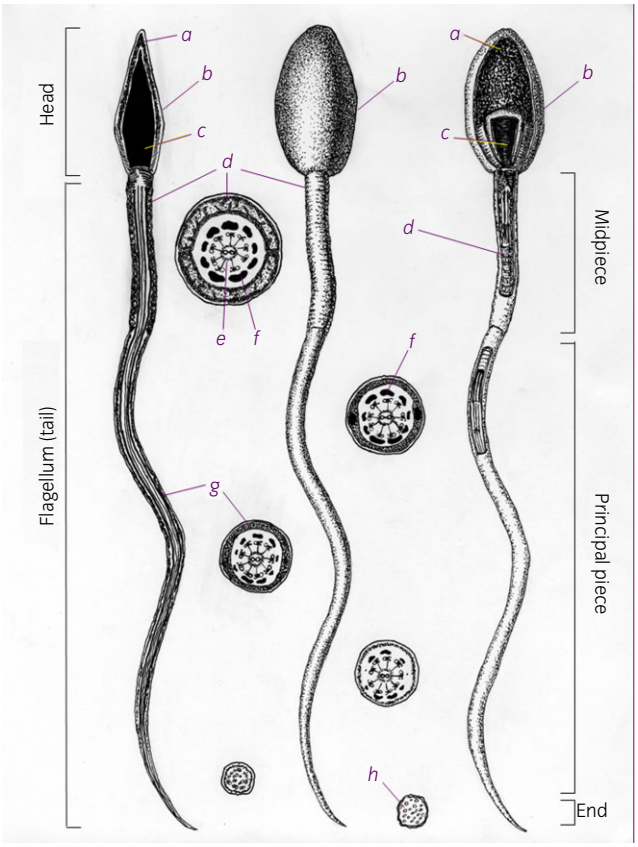

Fig 1: Drawings demonstrating magnified views of an equine spermatozoon, represented by an uncut view (centre), a mid-sagittal view (left), and a partially resected view (right). The various lengthwise divisions of the spermatozoon are represented as head, flagellum (tail), midpiece, principal piece, and end piece (end). a. acrosome, b. plasma membrane, c. nucleus, d. mitochondria, e. axoneme, f. outer dense fibres, g. fibrous sheath, h. axonemal microtubules (From Varner and Johnson (2007) *Proc. Am. Ass. Equine Practnrs*, **53**, 104–177, reproduced with permission of the American Association of Equine Practitioners.)

some species (e.g. the mouse, rat and hamster). Of the laboratory animal species, the equine spermatozoon most closely resembles that of the rabbit. The spermatozoal head is somewhat elliptical in shape, is flattened in one plane (dorsoventrally), and is thicker in the posterior portion of the head than in the apical portion (Fig 2) [5–7]. The nucleus, which occupies the majority of space within the head, contains the paternal genetic material. Specifically, the male genome is comprised of the X or Y chromosome and a haploid number of somatic chromosomes. This genetic material is packaged for delivery to the oocyte at fertilization, where 2 haploid (male and female) genomes are combined to produce a diploid offspring. The chromatin (i.e. the DNA and associated proteins) within the nucleus of the mature spermatozoon is highly condensed, resulting in a volume that may approach only 5–10% of that of a somatic cell. This packing is a result of a marked alteration in the composition and cross-linking of nucleoproteins which occurs during spermiogenesis (the terminal stage of spermatogenesis when spermatozoa are differentiated from round spermatids) and epididymal transit. The haploid genome of the round spermatid encodes for unique spermatozoal proteins, termed protamines, which predominate as nucleoproteins during spermatozoal maturation in the epididymis. The cysteine residues of these proteins establish intra- and inter-molecular disulfide linkages that result in compaction and stabilisation of the associated DNA. This design is thought to provide protection to the chromosomes during their perilous journey within the female reproductive tract, and to provide spermatozoa with a streamlined, spear-like structure, that facilitates mobility upon activation at the time of ejaculation and, later, aids in the penetration of the *zona pellucida* (ZP), a thick a cellular shell that surrounds the egg.

The nuclear envelope, consisting of a double membrane (each with a lipid bilayer), separates the contents of the nucleus from the surrounding cytoplasm. While the nuclear envelope is regularly perforated by nuclear pore complexes in somatic cells, such pores are absent over most of the spermatozoal nuclear envelope, i.e. in the area under the acrosome and in the post acrosomal region. The exception is the region of the *redundant nuclear envelope*, a portion of the nuclear envelope posterior to the chromatin that folds and extends back into the neck region. This portion of the nuclear envelope contains abundant hexagonally arranged pores. The caudal portion of the nuclear envelope forms a concavity, termed the *implantation fossa*, which is the site of attachment with the flagellum. This region of the nuclear envelope is overlain with a thick sheet of material, termed the *basal plate* (Fig 2).

The acrosome is a membrane-bound exocytotic organelle that overlies the rostral two-thirds of the nucleus, with a fit resembling that of a bathing cap. Anatomically, the membrane is subdivided into an inner acrosomal membrane, which is continuous with an outer acrosomal membrane. These connecting membranes enclose a narrow heterogeneous compartment, termed the acrosomal matrix. The inner acrosomal membrane is in close apposition with the nuclear envelope whereas the outer acrosomal membrane underlies the plasma membrane. These 2 membranes converge at the level of the *equatorial segment*. The acrosome originates from the Golgi apparatus in round spermatids during spermiogenesis. Refinement in acrosomal morphology and biochemical

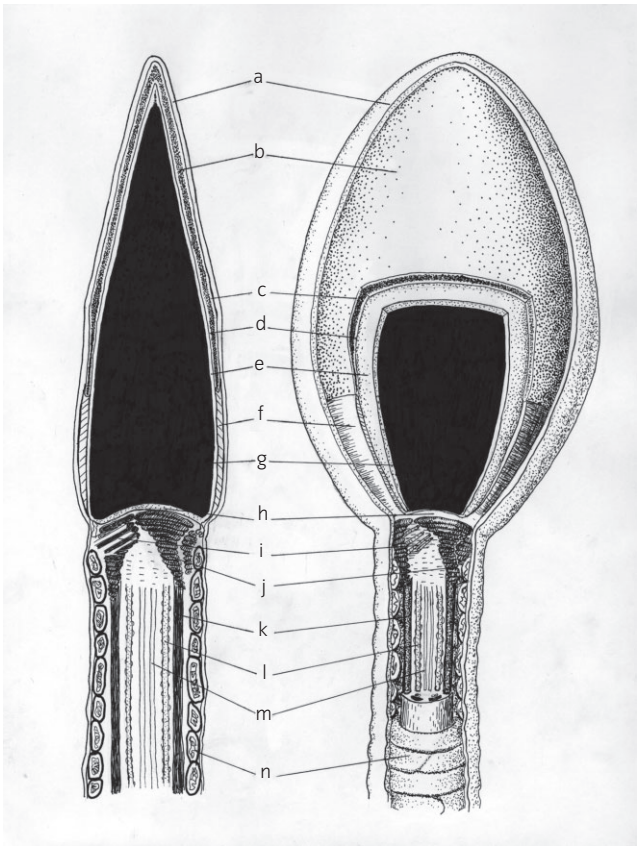

Fig 2: Further magnified illustrations of Fig 1, revealing midsagittal and partially resected views of equine spermatozoa. a. plasma membrane, b. acrosome, c. outer acrosomal membrane, d. inner acrosomal membrane, e. nuclear envelope, f. post acrosomal lamina, g. nucleus, h. basal lamina and underlying capitulum, i. proximal centriole, j. segmented column, k. outer dense fibres, l. outer doublets of axoneme, m. centre pair of microtubules within the axoneme, n. mitochondria. (From Varner and Johnson (2007) *Proc. Am. Ass. Equine Practnrs*, **53**, 104–177, reproduced with permission of the American Association of Equine Practitioners.)

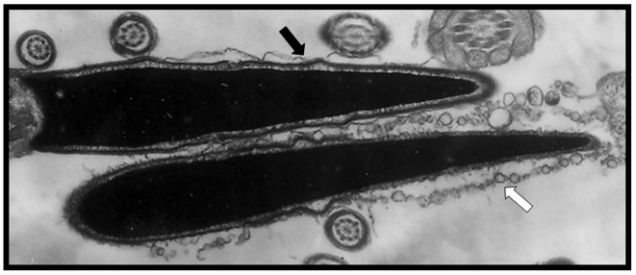

Fig 3: Transmission electron micrograph revealing sagittal views of adjacent equine spermatozoal heads. One spermatozoon has an intact acrosome (solid arrow) and one has undergone the acrosome reaction induced by the calcium ionophore, A23187 (open arrow). (From Varner and Johnson (2007) *Proc. Am. Ass. Equine Practnrs*, **53**, 104–177, reproduced with permission of the American Association of Equine Practitioners).

composition continue as spermatozoa traverse the epididymis [8]. In the mature spermatozoon, the acrosome contains a bountiful supply of active molecules, both within the acrosomal matrix and as components of the inner acrosomal membrane. These molecules, which include an assortment of protein receptors and hydrolytic enzymes, are thought to be important for adhesion to, and penetration of, the ZP, as well as spermatozoon–oolemma interactions. During the course of the acrosome reaction, the outer acrosomal membrane fuses with the overlying plasma membrane, thereby creating hybrid vesicles and pores that lead to release and exposure of acrosomal contents (Fig 3) [8]. Although the spermatozoal acrosome contains several enzymes characteristic of a typical cellular lysosome (in addition to those enzymes specific to the spermatozoon), the enzymes are not used within the cell as in autophagy for cellular organelle remodelling and renewing, or in heterophagy, as occurs in phagocytic cells.

The cytosolic compartment of the mature spermatozoal head is virtually free of organelles other than the acrosome, and the mature spermatozoon is generally considered to be transcriptionally quiescent. Nonetheless, the cytoplasm of the head region contains cytoskeletal proteins that are important to spermatozoal function. Structural changes occur in the cytoskeleton (both microfilaments and microtubules) during spermiogenesis that lead to the reshaping of the round spermatid head to an elongated form [9–12].

The connecting piece of the flagellum consists primarily of a *capitulum*, *segmented columns* of fibres, and the proximal and distal *centrioles*. The connecting piece serves to attach the flagellum to the head, and also functions to produce and stabilise some structural components of the flagellum. The capitulum articulates with the head at the level of the implantation fossa, attaching by fine filaments that connect the capitulum to the basal plate. The segmented columns anchor the dense fibres of the flagellum (Fig 2). The centrioles, oriented at right angles to each other, are involved in development of the connecting piece and the axoneme. The proximal centriole remains attached at the implantation fossa, but the distal centriole gives rise to the axoneme during tail development.

The primary structural elements of the flagellum include the *axoneme*, the *outer dense fibres*, the *fibrous sheath*, and the *mitochondria* (Figs 1, 2 and 4). The midpiece is characterised by the presence of an axoneme, an array of outer dense fibres, and an overlying sheath of helically arranged mitochondria. The midpiece joins the principal piece at the *annulus*, a point where the mitochondrial sheath is replaced with a fibrous sheath. The principal piece thereby consists of a centralised axoneme, outer dense fibres of variable length, and a fibrous sheath. The fibrous sheath terminates at the junction of the principal piece and end piece, with the end piece consisting of a small extension of the axoneme (or individually arranged acrosomal microtubules) past the termination site of the fibrous sheath. The entire flagellum is enveloped by a plasma membrane.

The axoneme is cylindrical array of *nine doublet microtubules* that surround 2 singlet microtubules (termed the *central pair*) connected by regularly spaced bridges, thus forming the '9 + 2' configuration that is characteristic of both cilia and flagella throughout the plant and animal kingdoms. The microtubules are composed primarily of the tubulin family of proteins. Each of the 9 outer microtubule doublets consists of an 'A' subunit, which is completely cylindrical and composed of 13 protofilaments, and a 'B' subunit, which is C-shaped and composed of 10–11 protofilaments (Fig 4). The A subunits serve as an anchor for the outer dynein arms and inner dynein arms which possess adenosine triphosphatase (ATPase) activity and generate the force required for axonemal and, thereby, flagellar motion through an attachment–detachment cycle between A- and B-subunits of adjacent doublets. Filamentous *nexin links* or *nexin arms* connect adjacent doublets, and *radial spokes* connect the axonemal doublets to a helical sheath surrounding the central pair of microtubules.

The outer dense fibres course from the connecting piece through the midpiece and into the principal piece. These fibrous structures are thought to provide structural support as well as passive elasticity during flagellar bending. Nine irregularly shaped outer dense fibres occupy sites overlying the 9 axonemal doublets along the entire length of the midpiece. The outer dense fibres taper at varying locations within the principal piece. Two of the outer dense fibres terminate in the proximal portion of the principal piece, and the structural support of these 2 fibres is replaced by inward extensions of the fibrous sheath.

The mitochondrial sheath forms near the end of spermiogenesis, and the mitochondria assume their final position in an end-to-end spiral arrangement of mitochondrial gyres around the outer dense fibres of the midpiece. The length of the midpiece, and the number of mitochondrial gyres, varies greatly among mammalian species, but is relatively constant within a given species. The equine spermatozoon contains 40–50 mitochondrial gyres. The order and symmetry of the mitochondria along

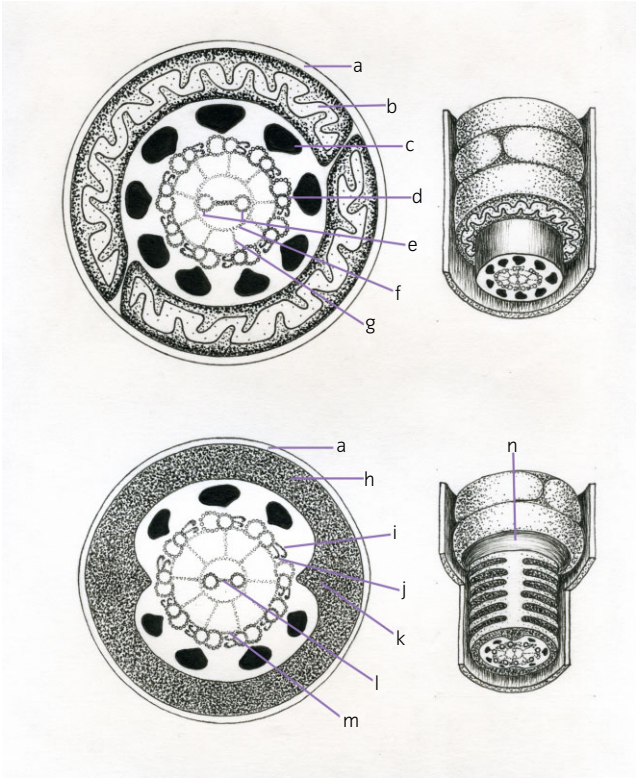

Fig 4: Magnified illustrations of cross sections through the middle piece (midpiece) and principal piece regions of equine spermatozoa. a. plasma membrane, b. mitochondria, c. outer dense fibre, d. axonemal doublet, e. central pair of axonemal microtubules, f. radial arm, g. fibrous sheath, h. annulus, i. outer dynein arm, j. inner dynein arm, k. longitudinal column of fibrous sheath, l. connecting bridge between central pair of axonemal microtubules, m. nexin links, n. sheath surrounding central pair of axonemal microtubules. (From Varner and Johnson (2007) *Proc. Am. Ass. Equine Practnrs*, **53**, 104–177, reproduced with permission of the American Association of Equine Practitioners.)

the midpiece may convey a functional effect, as disruptions in this organisation have been associated with reduced fertility of stallions [13].

The fibrous sheath extends along the entire length of the principal piece, i.e. from the annulus to the end piece. It is composed of 2 longitudinally arranged fibrous columns that are bridged by a series of interconnecting, circumferentially arranged fibrous ribs. The fibrous sheath is thought to provide rigid structural support and elasticity to the flagellum, but it also serves an important role as a scaffold for a variety of cell-signalling and metabolic events.

The axoneme, dynein arms, outer dense fibres, mitochondrial sheath, and fibrous sheath are often portrayed as the fundamental elements of the flagellum. While each is vital to flagellar function, one must also be aware that these elements are embedded in a network of other molecules that are equally important. Bending of the flagellum is caused by reciprocal sliding between doublet microtubules within the axoneme. The dynein arms are permanently attached to one doublet microtubule with arms extending to engage intermittently an adjacent doublet microtubule. When the crossbridges created by the dynein arms are complete between neighbouring doublets, the corresponding microtubules are prevented from sliding. Dynein is high molecular weight ATPase, so the dynein arms have the capacity to transform chemical energy into mechanical force. Each dynein arm has 3 adenosine triphosphate (ATP)-sensitive binding sites and all sites must bind with ATP for the dynein arm to detach from the microtubule, i.e. the crossbridge attachment–detachment cycle is dependent on binding and hydrolysis of ATP [14]. The conformational change in the dynein arms results in the force generation required for microtubule sliding. The microtubules of the axoneme enhance the rate of adenosine diphosphate release following the dephosphorylation to aid the reactivation step [15]. The nexin arms also undergo periods of displacement to allow sliding of microtubules [16]. The basic mechanism is similar to that of muscle myosin; however, the loss and rebinding of ATP products occurs at 2–3 orders of magnitude faster for dynein than for myosin [17]. Bending of the tail occurs during axonemal sliding because the microtubules are fixed at the base of the flagellum [18]. The passive elastic nature of the overlying outer dense fibres and fibrous sheath permits the flagellum to bend, and also provides elastic recoil.

Bending of the flagellum requires a patterned activation of microtubule sliding around the circumference, and along the length, of the cylindrical array of microtubules that compose the axoneme. This dynein-generated activity is regulated by the central pair of microtubules and their associated structures, collectively called the central apparatus. The asymmetry of components in the central apparatus forms the basis of a 'timing device' that creates the spatially timed sliding of various microtubules [19,20]. As such, the central apparatus both constrains and activates the dynein arms via communication through the radial spokes [16,21].

Spermatozoal metabolism

Spermatozoa are among the most highly specialised mammalian cells, with the roles of delivering paternal DNA and triggering activation of the oocyte. Given that the site of spermatozoal deposition is physically removed from the site of fertilisation, the ability to generate energy in the form of ATP for the function of motility is essential. In addition, the process of capacitation, whereby spermatozoa undergo the final maturational changes required to fertilise the oocyte, is a highly energy dependent process involving numerous modifications to motility patterns and membrane structure [22].

The 2 main metabolic pathways of energy production by spermatozoa are glycolysis and oxidative phosphorylation (OXPHOS). The enzymes associated with glycolysis are located in the principal piece of the tail, largely in association with the fibrous sheath, while OXPHOS occurs in the mitochondrial gyres located in the spermatozoal midpiece. Despite the fact that OXPHOS is significantly more efficient at producing ATP than glycolysis, spermatozoa from most species appear to depend predominantly on glycolysis for ATP production [23]. During spermiogenesis and epididymal transit, the male germ cell becomes transformed from a conventional spherical cell with a centrally placed nucleus to one of the most highly specialised cells in the body. During this transformation, the DNA in the sperm nucleus reaches the physical limits of compaction to achieve a quasicrystalline state that is incompatible with gene transcription [24]. Similarly a majority of the cytoplasm is discarded or resorbed during spermatozoal differentiation to remove a majority of the organelles (e.g. endoplasmic reticulum, ribosomes, Golgi apparatus) that regulate metabolism in somatic cells. As a result, spermatozoa are also translationally silent and largely devoid of intracellular energy reserves in the form of fat droplets, yolk granules and glycogen. As such, spermatozoa are highly dependent on their immediate extracellular environment for both the supply of energy substrates [25] and enzymatic activities that would normally be conducted intracellularly. An excellent example of such reliance on enzymes in the extracellular space that would, in normal somatic cells, be housed intracellularly, are the array of enzymes involved in protecting spermatozoa against oxidative stress. To compensate for their lack of cytoplasmic volume, spermatozoa are immersed in epididymal and seminal fluids that are endowed with the richest combination of antioxidant enzymes known to man, including several, such as glutathione peroxidase 5, that are unique to the male reproductive tract [26,27].

There has been considerable attention focused on the role of glycolysis in driving spermatozoal motility in a number of species. Because large polar molecules such as glucose cannot passively diffuse across the bilipid layer, the efficient, rapid uptake of this molecule is facilitated by a complex family of glucose transporters (GLUTs), which were first described by Kasahara and Hinkle in 1977 [28]. A number of GLUTs have been identified and characterised by their relative abilities to transport hexoses (e.g. glucose, fructose, mannitol), vitamins and amino sugars [29]. The first glucose transporter to be characterised was GLUT 1 and it is particularly highly expressed in erythrocytes [30]. Since the discovery of GLUT 1, a variety of other members of this family have been discovered with specialised distributions and functions. GLUTs 1, 2, 3, 4 and 5 have been identified on stallion spermatozoa, with the location of GLUTs 1, 2, 3 and 5 being the tail and the acrosome [31]. Whereas, GLUT 4 has no specific immunolabelling pattern, but its presence has been identified by Western blotting, with a weak band at 70/80 kDa in the horse [25]. These patterns of distribution suggest that glycolytic processes are involved in generating energy for the membrane modifications required for capacitation and the acrosome reaction. Should this be the case, then the distribution of GLUTs might be expected to change with the functional status of the cell (i.e. between noncapacitated and capacitated states), a phenomenon that has been reported in the dog, but does not occur in the horse [29]. At this stage, the significance of glycolysis for stallion spermatozoal ATP production in support of motility, capacitation or the acrosome reaction, has not been fully resolved. Nevertheless, it is already apparent that the stallion spermatozoon differs from other well-studied mammalian species, such as the mouse and man, in being heavily reliant on OXPHOS to meet its energy demands (Fig 5). Thus, in the presence of mitochondrial inhibitors, stallion spermatozoa rapidly lose velocity and ATP levels decline dramatically [32]. Although studies such as these highlight the relative contribution of OXPHOS to energy production in the spermatozoa of this species, deciphering the relative importance of glycolysis to stallion spermatozoal function has been somewhat more problematic. Many studies have attempted to quantify the relative contribution of glycolysis by inhibition of the process using the biologically inert glucose derivative 2-deoxyglucose. While this method does indeed result in a decrease in ATP, it is a 'blunt' instrument for dissecting energy pathways because depletion of ATP is due to both the inhibition of glycolysis and the futile consumption of ATP by hexokinase as this enzyme phosphorylates 2-deoxyglucose to generate 2-deoxyglucose-6-phosphate; the latter then accumulates in the cell because it cannot be further processed by the enzymes of the glycolytic pathway [30]. A more direct approach to the quantification of glycolytic processes would be to perform flux and product distribution measurements using ^{14}C-labelled glucose and evaluating the production of ^{14}C-labelled pyruvate and lactate in the presence and absence of mitochondrial inhibition.

The hypothesis that stallion spermatozoa depend predominantly on OXPHOS for energy generation has been developing for some time. This idea arose from observations of a nonconventional relationship between reactive oxygen species (ROS) and fertility in the stallion [33,34]. The source of ROS under these conditions is the mitochondrial electron

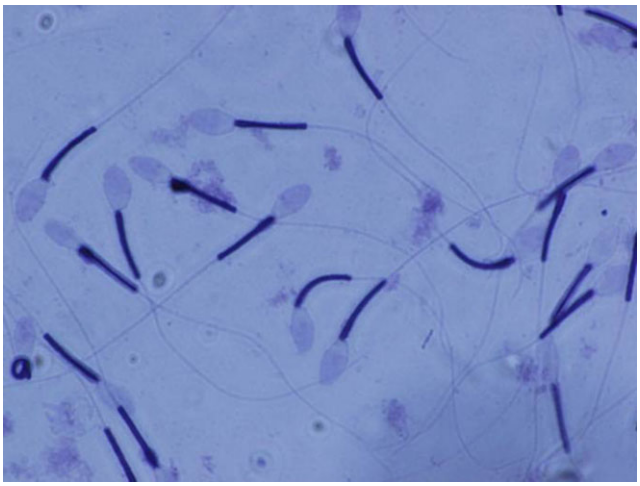

Fig 5: Stallion spermatozoa primarily utilise mitochondrial oxidative phosphorylation for the generation of adenosine triphosphate for motility. The mitochondria of the midpiece may be visualised by staining with nitroblue tetrazolium, which changes to a dark blue colour on reduction by oxidative phosphorylation-associated molecules.

transport chain, in which about 1–3% of O_2 reduced in the mitochondria during OXPHOS forms superoxide [35]. While the finding that stallion spermatozoa generate ROS is not novel, the general concept has always been that it is the nonviable or poor quality spermatozoa that generate the most ROS [36]. However, an alternative explanation is that rapidly metabolising spermatozoa from highly fertile stallions exhibit higher levels of OXPHOS activity and therefore present with elevated levels of ROS generation and lipid peroxidation.

Thus, while human clinical data have consistently demonstrated a negative correlation between male fertility and markers of oxidative stress in the spermatozoa [37,38], recent experimental results have turned this paradigm around in the stallion, revealing an inverse relationship between fertility and the percentage of live cells *without* oxidative damage [32]. In addition, ejaculated spermatozoa from matings that did result in conception (and were therefore considered to be more fertile) had lower vitality and a higher percentage of cells displaying ROS-induced damage upon arrival at the laboratory than spermatozoa from matings that did not result in a conception [32]. From these results, we hypothesise that during *in vitro* storage and transport of the samples to the laboratory, spermatozoa from the more fertile stallions, which are assumed to be more metabolically active, were becoming exhausted at a higher rate such that by the time that the assays were performed in the laboratory, these cells suffered an accelerated demise due to the accumulation of metabolic by-products, such as ROS and cytotoxic lipid aldehydes. Essentially, we propose that spermatozoa from highly fertile stallions 'live fast and die young'.

Following this observation, a set of experiments was designed to elucidate the mode of ATP generation by equine spermatozoa. Due to the introduction of artefacts following glycolytic inhibition [39], inhibition of OXPHOS was undertaken in a comparative study between equine spermatozoa and a species known to be highly dependent on glycolysis for spermatozoal motility (man). In this experiment OXPHOS inhibition resulted in over 70% reduction in velocity and a 75% reduction in ATP levels of equine spermatozoa, while human spermatozoal velocity and ATP remained unaffected. In addition, the greater efficiency of OXPHOS mediated ATP production by equine spermatozoa supported a higher velocity such that stallion spermatozoal velocity parameters were approximately 60% faster than those of human spermatozoa.

Relatively high ROS production by stallion spermatozoa appears to be a physiologically normal scenario brought about by superoxide leakage from the mitochondrial electron transport chain during OXPHOS [33], with a positive relationship between mitochondrial ROS production and spermatozoal velocity, leading to increased rates of lipid peroxidation [32] and, following prolonged storage, a loss of motility and vitality [40]. Stallion spermatozoa appear to have evolved an effective defence against the damaging effects of ROS, with an abundance of catalase compared with other species [41]. Thus, while oxidative stress is undoubtedly damaging to equine spermatozoa, in the short term, the particularly high levels of mitochondrial activity that characterises spermatozoal metabolism in this species may lead to counterintuitive positive correlations between ROS generation, lipid peroxide formation and fertility. Hence in a diagnostic context, the discovery of oxidative stress in stallion spermatozoa should be taken as a positive reflection of their metabolic activity. However, this tendency to generate ROS has negative connotations when it comes to storing these cells, since the prolonged generation of ROS in the absence of extracellular free radical and lipid aldehyde scavengers, will lead to irreversible oxidative damage to these cells, impairing DNA integrity and spermatozoal functionality. In recognition of such factors, several authors have attempted to improve stallion spermatozoal function following cryostorage through antioxidant supplementation but have obtained disappointing results [42,43] in contrast to the generally positive effects seen in human spermatozoa [44,45]. Clearly, further refinement of the antioxidants [46] that might be used to facilitate the long-term storage of stallion spermatozoa is required in the future.

The importance of understanding the modes by which stallion spermatozoa generate energy is apparent. Spermatozoal motility is not only lost as a consequence of lipid peroxidation due to ROS attack [14], but also due to the concomitant depletion of ATP [47]. With respect to the storage of stallion spermatozoa for artificial insemination, a better understanding of metabolic processes will allow tailoring of the media constituents to provide the correct energy sources (e.g. glucose vs. pyruvate) and carrier molecules to allow the unimpeded generation of ATP for optimal sperm motility, functionality and fertility. Given that mitochondrial metabolism is the source of the majority of ROS, a mitochondrial antioxidant that can effectively scavenge leaked superoxide may present the best option to reduce the downstream effects of ROS on spermatozoal function and DNA integrity. In addition, the diagnostic relevance of oxidative stress to stallion fertility must be re-assessed. While the effects of oxidative stress are never going to be beneficial, they may be indicative of an extremely fertile sample while remaining at subclinical levels. A major implication of this phenomenon is that semen assessments must be performed as close as possible to the time of collection to avoid ROS-induced artefacts.

Laboratory assays for assessing spermatozoal function

Given our improved understanding of the morphology and metabolism of stallion spermatozoa, what implications are there for the accurate diagnosis of spermatozoal function? Traditionally, the diagnosis of male infertility is based on a conventional semen profile, which provides an indirect assessment of the quality of the spermatogenic process by evaluating the number, morphology and motility of spermatozoa in the ejaculate. Such conventional criteria remain at the heart of diagnostic andrology even though several studies have indicated that such parameters only have diagnostic significance when severely impaired [48]. Importantly they do not have the diagnostic power required to resolve relative degrees of fertility, which is often required in the horse breeding industry, but only serve to identify the relatively small number of stallions where fertility is profoundly compromised because of fundamental defects in spermatogenesis. In order to improve the sensitivity of diagnostic tests for stallion fertility, emphasis has been placed on assessing the functionality of these cells *in vitro* with specific reference to their motility and their capacity to engage in the complex cascade of cellular interactions that culminate in fertilisation (Fig 6). In the following discussion, these aspects of sperm function are reviewed in terms of their biological evaluation and diagnostic significance.

Motility assessments

The diagnostic power of spermatozoal motility assessments has been improved by the introduction of computer aided semen analysis (CASA) systems to quantify objectively the various elements of spermatozoal movement. Such systems provide an objective measure of the quality of

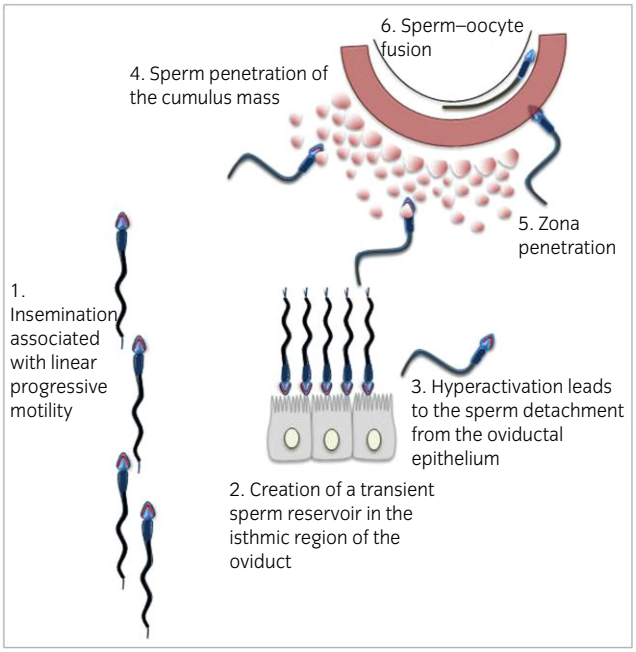

Fig 6: The key components of spermatozoal function in the stallion. 1) At insemination spermatozoa are inseminated into the female reproductive tract and immediately express a vigorous pattern of progressive motility that carries them through the intramural portion of the Fallopian tube and into the isthmic region. 2) In this location, the spermatozoa establish intimate contact with the epithelium and enter a state of relative quiescence forming a transient post insemination sperm store. 3) Signals associated with ovulation lead to the activation of hyperactivated motility in these spermatozoa causing them to break away from the oviductal epithelium and transit towards the ampulla of the Fallopian tube where fertilisation will occur. 4) As hyperactivated spermatozoa approach the oocyte, they respond to the high concentration of progesterone in the immediate vicinity of this cell by initiating the acrosome reaction. The latter may be driven to completion before the spermatozoa reach the *zona pellucida* and/or may be completed on spermatozoal binding to the *zona pellucida*. 5) The acrosome reacted spermatozoa then penetrate through the *zona pellucida* and enter the perivitelline space. 6) In this location, the plasma membrane overlying the equatorial segment of the fertilising spermatozoon binds to the vitelline membrane of the oocyte and initiates fusion.

spermatozoal motility by following the trajectory of the spermatozoal head in 2 dimensions. Although CASA systems can generate information of diagnostic significance, it is questionable as to whether the detailed movement characteristics of stallion spermatozoa generate more prognostic information than a consideration of percentage motility alone [49]. Furthermore, there is one important respect in which the simple application of CASA systems to ejaculated spermatozoa fails to give a comprehensive view of the quality of spermatozoal movement.

One of the most intriguing aspects of spermatozoal motility is the way in which its detailed form can change to meet the demands imposed by synchronising the arrival of fully capacitated spermatozoa with mature oocytes in the ampullae of the Fallopian tubes in preparation for fertilisation. In all Eutherian mammals, such synchronisation is achieved by creating a post insemination reservoir of spermatozoa in the isthmic region of the oviduct [50]. In this location, the spermatozoa remain in a quiescent state until a signal associated with ovulation induces their sudden activation and release (Fig 6). When spermatozoa are released from the isthmic store, their motility changes from the small amplitude symmetrical flagellar beat pattern characteristic of freshly ejaculated spermatozoa to a large amplitude, high frequency asymmetrical 'hyperactivated' beat pattern that is capable of generating the kind of propulsive forces needed to break away from the isthmic epithelium and penetrate the investments surrounding the egg. Hyperactivated movement is thus an extremely important aspect of spermatozoal function without which fertilisation cannot occur [51].

There are 2 major problems with the routine use of this criterion for diagnostic purposes. Firstly, although hyperactivation generates a characteristic figure-of-8 pattern of movement, it cannot be accurately captured by CASA machines because the latter uses algorithms that assume a 2-dimensional pseudosinusoidal pattern of movement. Such modelling of spermatozoal motion is ideal when it comes to capturing parameters such as 'amplitude of lateral head displacement' or 'straight line velocity' that are typical of the forward progressive movement expressed by freshly ejaculated spermatozoa. However, when spermatozoa are conducting a 3-dimensional figure-of-8 pattern of hyperactivated motility, these movement parameters are of limited value. The CASA systems can be adapted to give an approximate readout of the percentage of cells that are hyperactivated but it requires the calculation of additional spermatozoal movement criteria such as dancemean [amplitude of lateral head displacement/linearity × 100]. For human spermatozoa, the optimal combination of CASA parameters for measuring the size of the hyperactivated spermatozoal population has been defined as curvilinear velocity >90 μm/s, linearity <20%, dancemean >45.8 μm) after 3 h of incubation [52]. No such analysis has yet been conducted for equine spermatozoa.

The lack of CASA-based criteria for measuring hyperactivated motility in stallion spermatozoa may not be a major issue diagnostically because, while CASA parameters have the advantage of objectivity and consistency, the most effective method of scoring hyperactivation is still the direct visual classification of spermatozoa. This incidence of this pattern of movement can be rapidly assessed by a trained observer and readily distinguished from other related forms of movement such as 'transitional'. The latter is commonly exhibited in species, such as man, where capacitated spermatozoa exhibit multiphasic behaviour during which they transit in and out of hyperactivation, each burst of hyperactivated motility being separated from the next by a period of transitional movement characterised by large amplitude symmetrical flagellar waves. Careful training of technical staff to recognise these different patterns of movement is essential, because it is abundantly clear from the literature that not all authors publishing on hyperactivated motility have been scoring the same type of movement [52].

A second issue with spermatozoal hyperactivation is defining the conditions under which this activity is expressed. There is a general consensus that hyperactivated movement is a property expressed by capacitated spermatozoa, so any *in vitro* condition commensurate with capacitation should permit expression of this form of movement. Unfortunately, this is not the case. In some species such as the hamster, capacitation in simple defined media *in vitro* is associated with the synchronised expression of hyperactivation by the entire spermatozoal population in the absence of any further stimulation [53]. In the case of human spermatozoa however, hyperactivation is not invariably associated with the appearance of a capacitated state and even when it does appear, its expression tends to be transitory.

Defining the culture conditions that permit the consistent expression of hyperactivated movement is therefore a challenge for andrologists irrespective of species in which they are interested. In the case of the stallion, the problems experienced by some authors in capacitating these cells emphasises the difficulties that have been encountered in developing a robust diagnostic test of hyperctivated motility that will have prognostic value. One solution to this problem may be to simply bypass the biological processes that drive capacitation completely and directly trigger hyperactivated movement using a membrane stabiliser, such as procaine. By stabilising the plasma membrane, procaine impedes the activity of Ca/Mg ATPases responsible for maintaining low intracellular calcium levels. As a result, exposure to this reagent leads to a sudden increase in intracellular calcium levels and this change, in turn, elicits a hyperactivated form of movement. Procaine is an effective inducer of hyperactivated movement in all species that have been tested, including the stallion [54]. A hyperactivated response to this reagent indicates that the intracellular signalling machinery downstream of calcium influx is intact and functional and capable of generating large amplitude flagellar waves responsible for this form of motility. It does not, however, instruct us as to the competence of the spermatozoa to admit calcium at the appropriate time in response to the physiological triggers associated with ovulation. In order to develop more physiological assays of hyperactivated movement that more

accurately reflect the behaviour of a given population of spermatozoa *in vivo*, the molecular mechanisms responsible for regulating calcium entry under physiological circumstances need to be considered.

In a model animal species such as the mouse, hyperactivation can be readily elicited by cyclic adenosine monophosphate (cAMP) analogues, suggesting an involvement of protein phosphorylation in this process [55]. The targets for cAMP-induced phosphorylation include the sodium/hydrogen exchanger, sHNE, which leads to intracellular alkalinisation, and the potassium channel SLO3, which leads to membrane hyperpolarisation in the flagellum. Cytoplasmic alkalinisation and membrane hyperpolarisation set up the entry channel for extracellular calcium, CatSper [55]. The spontaneous entry of calcium through this channel ultimately leads to the expression of hyperactivated movement in capacitated mouse spermatozoa exhibiting high levels of tyrosine phosphorylation in the flagellum [55]. The effect may be direct or, more probably, reflect the filling of the intracellular calcium stores, which become sensitised to calcium induced-calcium release during capacitation via mechanisms that may involve nitric oxide-mediated S-nitrosylation of ryanodine receptors and/or cAMP mediated processes [56]. In certain species, such as the human, hyperactivation requires a specific stimulus to induce calcium entry through the CatSper channel in the flagellum. In this case, the specific stimulus is provided by progesterone, which is capable of eliciting significant levels of hyperactivation in spermatozoa that have been primed by cAMP [57].

In the case of stallion spermatozoa, we are still a long way from being able to define a set of physiologically relevant *in vitro* conditions whereby we can assess the competence of these cells to express a hyperactivated pattern of movement. Although we know that procaine can elicit this pattern of activity, and that procaine-elicited hyperactivation is supportive of high levels of fertilisation [54,58], the factors that normally regulate calcium entry into these cells are unclear. Indeed, even the importance of calcium in this process has recently been called into question as far as stallion spermatozoa are concerned [59]. Until the fundamental biochemical mechanisms that lead to the expression of equine hyperactivated motility *in vivo* are resolved, meaningful assays of this attribute of spermatozoal behaviour for diagnostic purposes will be difficult, if not impossible, to establish.

The acrosome reaction and sperm–oocyte fusion

If hyperactivation is generally considered to be the consequence of calcium entry into the spermatozoal flagellum, the acrosome reaction is thought to be the biological manifestation of calcium entry into the spermatozoal head (Fig 6). Robust methods of measuring acrosome integrity have been developed, employing lectins that are capable of specifically labelling the outer acrosomal membrane or the acrosomal contents [60]. In terms of fundamental cell biology, the acrosome reaction is a highly unusual exocytotic event. Relative to most other secretory processes, it is extremely slow, taking minutes to achieve what an islet cell or a synapse can accomplish in a matter of seconds. Secondly, during the acrosome reaction, the membranes defining the acrosomal vesicle, the plasma and outer acrosomal membranes, are lost from the spermatozoa long before the contents of the acrosomal vesicle are dispersed [61]. Thus the most sensitive method for detecting the acrosome reaction involves the use of labelled lectins from sources such as *Arachis hypogaea*, to monitor the status of the outer acrosomal membrane [61]. Another important aspect of acrosome reaction assays is that they must carefully distinguish a physiological acrosome reaction from pathological acrosomal loss. The latter is often observed with stallion spermatozoa particularly following cryostorage; however, physiological acrosome reactions are much harder to elicit. Differentiating true acrosome reactions from pathological acrosomal loss can be accomplished by incorporating into the assay a method for measuring spermatozoal vitality. For example, lectin staining can be conveniently combined with the hypo-osmotic swelling test to provide a simple, convenient and sensitive means of monitoring the acrosome reaction in populations of live spermatozoa [60].

The major problem that remains in developing diagnostic tests featuring the acrosome reaction is that, like hyperactivation, a set of conditions needs to be defined under which this process can be monitored in a physiologically meaningful manner. There is a general consensus that spermatozoa must have achieved a capacitated state to acrosome react, so conditions that favour capacitation (presence of extracellular bicarbonate and high levels of tyrosine phosphorylation) will promote the acrosome reaction *in vitro*. However, there is no current consensus for the physiological activation of this process *in vivo* or *in vitro*.

A decade ago, the physiological trigger for the acrosome reaction was deemed to be a protein constituent of the ZP, ZP3. However, attempts to build robust assays of acrosomal exocytosis using recombinant ZP3 as the trigger were never highly successful [62]. Acrosome reactions could be induced with such material but both the incidence and the speed of the process was not consistent with the *in vivo* situation, where we know that acrosome reactions can take place within minutes of the spermatozoa arriving at the surface of the egg [62]. Recent data suggest that ZP2 may in fact be the physiological inducer of the acrosome reaction [63]. Other data suggest that, *in vivo*, the spermatozoa may not acrosome react on the zona surface at all but may, in fact, acrosome react as cells are traversing the cumulus mass, possibly in response to the calcium influx mediated by progesterone [64]. Of course, none of these mechanisms are mutually exclusive and it is entirely possible that there is significant redundancy in the mechanisms responsible for triggering the acrosome reaction, such that progesterone, ZP glycoproteins and perhaps other factors [65], may be part of the array of biological triggers for this process *in vivo* [66].

The recognised ability of progesterone to induce acrosome reactions in equine spermatozoa and the fact that the acrosome reaction rates generated in the presence of this biological agonist *in vitro* are reflective of fertility rates *in vivo* [67,68], encourage belief that bioassays of acrosomal exocytosis are an achievable goal in this species. Similarly, recent studies demonstrating the use of bicarbonate rich culture media supplemented with methyl-β-cyclodextrin, an efficient cholesterol-withdrawing agent, to drive stallion spermatozoal capacitation and promote sperm–zona interaction is another important stepping stone towards the creation of *in vitro* systems for the diagnosis of spermatozoal function in the stallion [69]. The ultimate goal of such studies must be to define a set of conditions that resemble as closely as possible the array of physiological triggers for the acrosome reaction to which spermatozoa are exposed *in vivo*. Using such conditions, it should also be possible to generate meaningful data on other aspects of spermatozoal function dependent on capacitation including sperm–zona interaction (using bovine oocytes) and the generation of a fusogenic equatorial segment capable of initiating fusion with the vitelline membrane of the oocyte (using zona-free hamster oocytes) [70].

Ultimately, the development of an array of functional assays for assessing hyperactivated motility, acrosomal exocytosis, sperm–zona binding and sperm–oocyte fusion will be invaluable to helping us to categorise the specific nature of defective spermatozoal function in subfertile stallions. Using such methods, we shall be able to determine whether all elements of spermatozoal function are lost in an integrated and coordinated manner suggestive of a common cause, or whether particular aspects of spermatozoal function are lost more readily than others. Only when we are in possession of such knowledge can we reasonably address the critical issue of aetiology and the introduction of appropriate preventative strategies. Furthermore, once the aetiology of defective spermatozoal function has been elucidated in stallions, it should be possible to replace the time consuming and difficult-to-standardise bioassays described in this review with more targeted, biochemical assays that are amenable to the development of test kits that can be used on site, to give a rapid and robust assessment of the relative fertilising potential of individual stallions.

Closing comments

As seen by the information provided in the previous sections, we continue to garner a deeper understanding of spermatozoal structure and function. We have gained considerable insights from work conducted in man and laboratory animals. An appreciation of the molecular basis of spermatozoal function, and spermatozoal–oviductal and spermatozoon–oocyte interactions, will undoubtedly lead to many practical applications in the clinical front, such as assembly of a battery of in-depth laboratory tests to assess spermatozoal function; expanded treatment options for subfertile stallions; improved methods for preservation of semen; and heightened applications for assisted

reproductive technologies such as conventional *in vitro* fertilisation. Incorporation of such in-depth tests for semen evaluation does not replace, or diminish the value of, the classical measurements of spermatozoal motility or morphology, and these 2 methods are likely to remain the hallmarks for semen evaluation for years to come. However, a better understanding of the molecular mechanisms regulating spermatozoal development, metabolism and function will be likely to lead to new diagnostic techniques and therapeutic strategies for reduced fertility in stallions and may possibly translate to methodologies designed to curb gonadal ageing or improve methods for preservation of semen. Undoubtedly, future generations will look beyond descriptive morphology and motility in characterising structural and functional features and deficiencies of spermatozoa. The bottom line is that the more we learn, the more informed we can be in decision making regarding diagnostic and therapeutic strategies as they relate to spermatozoal function and reproductive health of stallions. It becomes incumbent upon us, as clinicians and academicians, to convert these opportunities into practical applications. Further scrutiny and standardisation of such tests is the key to success.

Authors' declaration of interests

The Grayson-Jockey Club Research Foundation commissioned this article as part of a series summarising areas relating to their priorities for research funding. Dr Varner and other workers at his institute hold current and previous research grants funded by the GJCRF and Dr Varner has served on GCJRF advisory committees.

Ethical animal research

This article is a review of the literature and does not involve experimental animals.

Sources of funding

None.

Authorship

Each author has contributed sections in this review article and all approved the final manuscript.

References

1. Rigby, S., Derczo, S., Brinsko, S.P., Blanchard, T.L., Taylor, T., Forrest, D.W. and Varner, D.D. (2000) Oviductal sperm numbers following proximal uterine horn or uterine body insemination. *Proc. Am. Ass. Equine Practnrs.* **46**, 332-334.
2. Cummins, J.M. and Woodall, P.F. (1985) On mammalian sperm dimensions. *J. Reprod. Fertil.* **75**, 153-175.
3. Bielanski, W. and Kaczmarski, F. (1979) Morphology of spermatozoa in semen from stallions of normal fertility. *J. Reprod. Fertil. Suppl.* **27**, 39-45.
4. Dott, H.M. (1975) Morphology of stallion spermatozoa. *J. Reprod. Fertil. Suppl.* **23**, 41-46.
5. Varner, D.D., Blanchard, T.L., Brinsko, S.P., Love, C.C., Taylor, T.S. and Johnson, L. (2000) Techniques for evaluating selected reproductive disorders of stallions. *Anim. Reprod. Sci.* **60-61**, 493-509.
6. Johnson, L., Amann, R.P. and Pickett, B.W. (1978) Scanning electron and light microscopy of the equine seminiferous tubule. *Fertil. Steril.* **29**, 208-215.
7. Johnson, L. (1991) Spermatogenesis. In: *Reproduction in Domestic Animals*, Ed: P.T. Cupps, Academic Press, San Diego. pp 173-219.
8. Gerton, G.L. (2002) Function of the sperm acrosome. In: *Fertilization*, Ed: D.M. Hardy, Academic Press, San Diego. pp 265-302.
9. Kierszenbaum, A.L. and Tres, L.L. (2004) The acrosome-acroplaxome-manchette complex and the shaping of the spermatid head. *Arch. Histol. Cytol.* **67**, 271-284.
10. Toshimori, K. and Ito, C. (2003) Formation and organization of the mammalian sperm head. *Arch. Histol. Cytol.* **66**, 383-396.
11. Tachibana, M., Terada, Y., Murakawa, H., Murakami, T., Yaegashi, N. and Okamura, K. (2005) Dynamic changes in the cytoskeleton during human spermiogenesis. *Fertil. Steril.* **84**, 1241-1247.
12. Holstein, A.F. and Roosen-Runge, E.C. (1981) *Atlas of Human Spermatogenesis*, Grosse Verlag, Berlin.
13. Love, C.C., Varner, D.D. and Thompson, J.A. (2000) Intra- and inter-stallion variation in sperm morphology and their relationship with fertility. *J. Reprod. Fertil. Suppl.* **56**, 93-100.
14. Shimizu, T. and Johnson, K.A. (1983) Kinetic evidence for multiple dynein ATPase sites. *J. Biol. Chem.* **258**, 13841-13846.
15. Holzbaur, E.L. and Johnson, K.A. (1989) Microtubules accelerate ADP release by dynein. *Biochemistry* **28**, 7010-7016.
16. Gagnon, C. (1995) Regulation of sperm motility at the axonemal level. *Reprod. Fertil. Dev.* **7**, 847-855.
17. Johnson, K.A. (1985) Pathway of the microtubule-dynein ATPase and the structure of dynein: a comparison with actomyosin. *Annu. Rev. Biophys. Biophys. Chem.* **14**, 161-188.
18. Fujimura, M. and Okuno, M. (2006) Requirement of the fixed end for spontaneous beating in flagella. *J. Exp. Biol.* **209**, 1336-1343.
19. Nakano, I., Kobayashi, T., Yoshimura, M. and Shingyoji, C. (2003) Central-pair-linked regulation of microtubule sliding by calcium in flagellar axonemes. *J. Cell Sci.* **116**, 1627-1636.
20. Wargo, M.J. and Smith, E.F. (2003) Asymmetry of the central apparatus defines the location of active microtubule sliding in *Chlamydomonas* flagella. *Proc. Natl. Acad. Sci. U. S. A.* **100**, 137-142.
21. Porter, M.E. and Johnson, K.A. (1989) Dynein structure and function. *Annu. Rev. Cell Biol.* **5**, 119-151.
22. Flesch, F.M. and Gadella, B.M. (2000) Dynamics of the mammalian sperm plasma membrane in the process of fertilization. *Biochim. Biophys. Acta Rev. Biomembr.* **1469**, 197-235.
23. Storey, B.T. (2008) Mammalian sperm metabolism: oxygen and sugar, friend and foe. *Int. Dev. Biol.* **52**, 427-437.
24. Miller, D., Brinkworth, M. and Iles, D. (2010) Paternal DNA packaging in spermatozoa: more than the sum of its parts? DNA, histones, protamines and epigenetics. *Reproduction* **139**, 287-301.
25. Bucci, D., Rodriguez-Gil, J.E., Vallorani, C., Spinaci, M., Galeati, G. and Tamanini, C. (2011) GLUTs and mammalian sperm metabolism. *J. Androl.* **32**, 348-355.
26. Vernet, P., Aitken, R.J. and Drevet, J.R. (2004) Antioxidant strategies in the epididymis. *Mol. Cell. Endocrinol.* **216**, 31-39.
27. Aitken, R.J. and Curry, B.J. (2011) Redox regulation of human sperm function: from the physiological control of sperm capacitation to the etiology of infertility and DNA damage in the germ line. *Antioxid. Redox Signal.* **14**, 367-381.
28. Kasahara, M. and Hinkle, P.C. (1977) Reconstitution and purification of the d-glucose transporter from human erythrocytes. *J. Biol. Chem.* **252**, 7384-7390.
29. Angulo, C., Rauch, M.C., Droppelmann, A., Reyes, A.M., Slebe, J.C., Delgado-López, F., Guaiquil, V.H., Vera, J.C. and Concha, I.I. (1998) Hexose transporter expression and function in mammalian spermatozoa: cellular localization and transport of hexoses and vitamin C. *J. Cell. Biochem.* **71**, 189-203.
30. Mueckler, M., Caruso, C., Galdwin, S.A., Panico, M., Blench, I., Morris, H.R., Allard, W.J., Lienhard, G.E. and Lodish, H.F. (1985) Sequence and structure of a human glucose transporter. *Science* **229**, 941-945.
31. Bucci, D., Isani, G., Spinaci, M., Tamannini, C., Mari, G., Zambelli, D. and Galeati, G. (2010) Comparative immunolocalization of GLUTs 1, 2, 3 and 5 in boar, stallion and dog spermatozoa. *Reprod. Domest. Anim.* **45**, 315-322.
32. Gibb, Z., Lambourne, S.R. and Aitken, R.J. (2014) The paradoxical relationship between stallion fertility and oxidative stress. *Biol. Reprod.* in press.
33. Ortega Ferrusola, O., González Fernández, L., Salazar Sandoval, C., Macías García, B., Rodríguez Martínez, H., Tapia, J.A. and Peña, F.J. (2010) Inhibition of the mitochondrial permeability transition pore reduces 'apoptosis like' changes during cryopreservation of stallion spermatozoa. *Theriogenology* **74**, 458-465.
34. Morrell, J.M., Johannisson, A., Dalin, A.M., Hammar, L., Sadebert, T. and Rodriguez-Martinez, H. (2008) Sperm morphology and chromatin integrity in Swedish warmblood stallions and their relationship to pregnancy rates. *Acta Vet. Scand.* **50**. doi:10.1186/1751-0147-50-2
35. Halliwell, B. and Gutteridge, J.M.C. (2003) *Free Radicals in Biology and Medicine*, 3rd edn, Oxford University Press, Oxford.
36. Ball, B.A., Vo, A.T. and Baumber, J. (2001) Generation of reactive oxygen species by equine spermatozoa. *Am. J. Vet. Res.* **62**, 508-515.

37. Aitken, R.J. and Clarkson, J.S. (1987) Cellular basis of defective sperm function and its association with the genesis of reactive oxygen species by human spermatozoa. *J. Reprod. Fertil.* **81**, 459-469.
38. Jones, R., Mann, T. and Sherins, R. (1979) Peroxidative breakdown of phospholipids in human spermatozoa, spermicidal properties of fatty acid peroxides, and protective action of seminal plasma. *Fertil. Steril.* **31**, 531-537.
39. Ford, W.C. (2006) Glycolysis and sperm motility: does a spoonful of sugar help the flagellum go round? *Hum. Reprod. Update* **12**, 269-274.
40. Aitken, R.J., Gibb, Z., Mitchell, L.A., Lambourne, S.R., Connaughton, H.S. and De Iuliis, G.N. (2012) Sperm motility is lost in vitro as a consequence of mitochondrial free radical production and the generation of electrophilic aldehydes but can be significantly rescued by the presence of nucleophilic thiols. *Biol. Reprod.* **87**, 1-11.
41. Ball, B.A., Gravance, C.G., Medina, V., Baumber, J. and Liu, I.K. (2000) Catalase activity in equine semen. *Am. J. Vet. Res.* **61**, 1026-1030.
42. Baumber, J., Ball, B.A. and Linfor, J.J. (2005) Assessment of the cryopreservation of equine spermatozoa in the presence of enzyme scavengers and antioxidants. *Am. J. Vet. Res.* **66**, 772-779.
43. Morillo-Rodríguez, A., Macías-García, B., Tapia, J.A., Ortega-Ferrusola, C. and Peña, F.J. (2012) Consequences of butylated hydroxytoluene in the freezing extender on post-thaw characteristics of stallion spermatozoa *in vitro*. *Andrologia* **44**, *Suppl.* **1**, 688-695.
44. Thomson, L.K., Fleming, S.D., Aitken, R.J., De Iuliis, G.N., Zieschang, J.A. and Clark, A.M. (2009) Cryopreservation-induced human sperm DNA damage is predominantly mediated by oxidative stress rather than apoptosis. *Hum. Reprod.* **24**, 2061-2070.
45. Moubasher, A.E., El Din, A.M., Ali, M.E., El-sherif, W.T. and Gaber, H.D. (2013) Catalase improves motility, vitality and DNA integrity of cryopreserved human spermatozoa. *Andrologia* **45**, 135-139.
46. da Silva, C.M., Macías-García, B., Miró-Morán, A., González-Fernández, L., Morillo-Rodriguez, A., Ortega-Ferrusola, C., Gallardo-Bolaños, J.M., Stilwell, G., Tapia, J.A. and Peña, F.J. (2011) Melatonin reduces lipid peroxidation and apoptotic-like changes in stallion spermatozoa. *J. Pineal Res.* **51**, 172-179.
47. de Lamirande, E., Jiang, H., Zini, A., Kodama, H. and Gagnon, C. (1997) Reactive oxygen species and sperm physiology. *Rev. Reprod.* **2**, 48-54.
48. Graham, J.K. (1996) Analysis of stallion semen and its relation to fertility. *Vet. Clin. North Am. Equine Pract.* **12**, 119-130.
49. Love, C.C. (2011) Relationship between sperm motility, morphology and the fertility of stallions. *Theriogenology* **76**, 547-557.
50. Hunter, R.H. (2012) Components of oviduct physiology in Eutherian mammals. *Biol. Rev. Camb. Philos. Soc.* **87**, 244-255.
51. Bedford, J.M. (1983) Significance of the need for sperm capacitation before fertilization in eutherian mammals. *Biol. Reprod.* **28**, 108-120.
52. Sukcharoen, N., Keith, J., Irvine, D.S. and Aitken, R.J. (1995) Definition of the optimal criteria for identifying hyperactivated human spermatozoa at 25 Hz using *in-vitro* fertilization as a functional end-point. *Hum. Reprod.* **10**, 2928-2937.
53. White, D.R. and Aitken, R.J. (1989) Relationship between calcium, cAMP, ATP and intracellular pH and the capacity to express hyperactivated motility by hamster spermatozoa. *Gamete Res.* **22**, 163-178.
54. Ortgies, F., Klewitz, J., Görgens, A., Martinsson, G. and Sieme, H. (2012) Effect of procaine, pentoxifylline and trolox on capacitation and hyperactivation of stallion spermatozoa. *Andrologia* **44**, *Suppl.* **1**, 130-138.
55. Harayama, H. (2013) Roles of intracellular cyclic AMP signal transduction in the capacitation and subsequent hyperactivation of mouse and boar spermatozoa. *J. Reprod. Dev.* **59**, 421-430.
56. Alasmari, W., Costello, S., Correia, J., Oxenham, S.K., Morris, J., Fernandes, L., Ramalho-Santos, J., Kirkman-Brown, J., Michelangeli, F., Publicover, S. and Barratt, C. (2013) Ca^{2+} signals generated by CatSper and Ca^{2+} stores regulate different behaviors in human sperm. *J. Biol. Chem.* **288**, 6248-6258.
57. Sagare-Patil, V., Vernekar, M., Galvankar, M. and Modi, D. (2013) Progesterone utilizes the PI3K-AKT pathway in human spermatozoa to regulate motility and hyperactivation but not acrosome reaction. *Mol. Cell. Endocrinol.* **374**, 82-91.
58. McPartlin, L.A., Suarez, S.S., Czaya, C.A., Hinrichs, K. and Bedford-Guaus, S.J. (2009) Hyperactivation of stallion sperm is required for successful *in vitro* fertilization of equine oocytes. *Biol. Reprod.* **81**, 199-206.
59. Loux, S.C., Crawford, K.R., Ing, N.H., González-Fernández, L., Macías-García, B., Love, C.C., Varner, D.D., Velez, I.C., Choi, Y.H. and Hinrichs, K. (2013) CatSper and the relationship of hyperactivated motility to intracellular calcium and pH kinetics in equine sperm. *Biol. Reprod.* **89**, 123.
60. Aitken, R.J., Buckingham, D. and Huang, G.-F. (1993) Analysis of the responses of human spermatozoa to A23187 employing a novel technique for assessing the acrosome reaction. *J. Androl.* **14**, 132-141.
61. Aitken, R.J. and Brindle, J.P. (1993) Analysis of the ability of three probes targeting the outer acrosomal membrane or acrosomal contents to detect the acrosome reaction in human spermatozoa. *Hum. Reprod.* **8**, 1663-1669.
62. Van Duin, M., Polman, J.E.M., De Breet, I.T.M., van Ginneken, K., Bunschoten, H., Grootenhuis, A., Brindle, J. and Aitken, R.J. (1994) Recombinant human *zona pellucida* protein ZP3 produced by Chinese hamster ovary cells induces the human sperm acrosome reaction and promotes sperm-egg fusion. *Biol. Reprod.* **51**, 607-617.
63. Avella, M.A., Xiong, B. and Dean, J. (2013) The molecular basis of gamete recognition in mice and humans. *Mol. Hum. Reprod.* **19**, 279-289.
64. Avella, M.A. and Dean, J. (2011) Fertilization with acrosome-reacted mouse sperm: implications for the site of exocytosis. *Proc. Natl. Acad. Sci. U. S. A.* **108**, 19843-19844.
65. Joshi, C.S., Khan, S.A. and Khole, V.V. (2014) Regulation of acrosome reaction by Liprin α3, LAR and its ligands in mouse spermatozoa. *Andrology* **2**, 165-174.
66. Aitken, R.J. and Nixon, B. (2013) Sperm capacitation: a distant landscape glimpsed but unexplored. *Mol. Hum. Reprod.* **19**, 785-793.
67. McPartlin, L.A., Littell, J., Mark, E., Nelson, J.L., Travis, A.J. and Bedford-Guaus, S.J. (2008) A defined medium supports changes consistent with capacitation in stallion sperm, as evidenced by increases in protein tyrosine phosphorylation and high rates of acrosomal exocytosis. *Theriogenology* **69**, 639-650.
68. Meyers, S.A., Overstreet, J.W., Liu, I.K. and Drobnis, E.Z. (1995) Capacitation *in vitro* of stallion spermatozoa: comparison of progesterone-induced acrosome reactions in fertile and subfertile males. *J. Androl.* **16**, 47-54.
69. Bromfield, E.G., Aitken, R.J., Gibb, Z., Lambourne, S.R. and Nixon, B. (2013) Capacitation in the presence of methyl-β-cyclodextrin results in enhanced *zona pellucida*-binding ability of stallion spermatozoa. *Reproduction* **147**, 153-166.
70. Landim-Alvarenga, F.C., Alvarenga, M.A., Seidel, G.E. Jr, Squires, E.L. and Graham, J.K. (2001) Penetration of zona-free hamster, bovine and equine oocytes by stallion and bull spermatozoa pretreated with equine follicular fluid, dilauroylphosphatidylcholine or calcium ionophore A23187. *Theriogenology* **56**, 937-953.